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Evidence for horizontal gene transfer and separation of effector recognition from effector function revealed by analysis of effector genes shared between cape gooseberry- and tomato-infecting formae speciales of *Fusarium oxysporum*

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SUMMARY

RNA sequencing (RNAseq) reads from cape gooseberry plants (*Physalis peruviana*) infected with *Fusarium oxysporum* f. sp. *physali* (*Foph*) were mapped against the lineage-specific transcriptome of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) to look for putative effector genes. Homologues of *Fol SIX1* (designated *SIX1a* and *SIX1b*), *SIX7*, *SIX10*, *SIX12*, *SIX15* and *Ave1* were identified. The near identity of the *Foph* and *Fol SIX7*, *SIX10* and *SIX12* genes and their intergenic regions suggest that this gene cluster may have undergone recent lateral transfer. *Foph SIX1a* and *SIX1b* were tested for their ability to complement a *SIX1* knockout mutant of *Fol*. This mutant shows reduced pathogenicity on susceptible tomato plants, but is able to infect otherwise resistant tomato plants carrying the *I-3* gene for Fusarium wilt resistance (*SIX1* corresponds to *Avr3*). Neither *SIX1a* nor *SIX1b* could restore full pathogenicity on susceptible tomato plants, suggesting that any role they may play in pathogenicity is likely to be specific to cape gooseberry. *SIX1b*, but not *SIX1a*, was able to restore avirulence on tomato plants carrying *I-3*. These findings separate the recognition of SIX1 from its role as an effector and suggest direct recognition by I-3. A hypervariable region of *SIX1* undergoing diversifying selection within the *F. oxysporum* species complex is likely to play an important role in SIX1 recognition. These findings also indicate that *I-3* could potentially be deployed as a transgene in cape gooseberry to protect this emerging crop from *Foph*. Alternatively, cape gooseberry germplasm could be explored for *I-3* homologues capable of providing resistance to *Foph*.

Keywords: *Fusarium oxysporum* f. sp. *lycopersici*, *Fusarium oxysporum* f. sp. *physali*, Fusarium wilt, mobile pathogenicity chromosome, *Physalis peruviana*, secreted in xylem genes, *Solanum lycopersicum*.

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INTRODUCTION

Physalis peruviana, commonly known as cape gooseberry, is a solanaceous plant native to tropical South America, typically growing in the Andes at 2000 m. Its economic value has grown in the last three decades as a result in part of its nutritional and medicinal properties (Franco *et al.*, 2007; Martinez *et al.*, 2010; Ramadan, 2011). Cape gooseberry represents the second most important fruit export in Colombia, worth approximately US$30 million per annum (Barrero *et al.*, 2012). This export income is threatened by vascular wilt disease of cape gooseberry caused by a newly discovered forma specialis of the fungus *Fusarium oxysporum*, here designated *Fusarium oxysporum* f. sp. *physali* (*Foph*). This disease was first described in 2005 (Estupiñán Rodríguez *et al.*, 2007) and has become one of the most important disease problems emerging in cape gooseberry in Colombia. In 2011, losses in production of 90% were reported (unofficially) in the central Cundinamarca Department of Colombia (Barrero *et al.*, 2012). As a consequence, producers moved to other places in the same region, spreading contaminated plant material and seeds (Barrero *et al.*, 2012; Osorio-Guarín *et al.*, 2016). There is therefore an urgent need to better understand this pathogen and to develop better strategies for the management of the disease it causes.

Pathogenic fungi in the *F. oxysporum* species complex are the causal agents of vascular wilt disease in many plants, including economically important crop plants, such as banana, cotton, melon and tomato (Michielse and Rep, 2009), in addition to cape gooseberry. The interaction between tomato (*Solanum lycopersicum*) and *F. oxysporum* f. sp. *lycopersici* (*Fol*) is one of the best-studied *F. oxysporum* pathosystems (Takken and Rep, 2010) and is ideal as a model system to study infection of plants in the Solanaceae. Similarly, the interaction between *Arabidopsis thaliana* and *F. oxysporum* f. sp. *conglutinans* (*Foc*) is a model pathosystem to study infection of plants in the Brassicaceae (Berrocal-Lobo and Molina, 2004; Ospina-Giraldo *et al.*, 2003; Thatcher *et al.*, 2012).

The *Fol* and *Foc* genomes are amongst the largest *Fusarium* genomes sequenced, at 61 Mb and 55 Mb, respectively, compared with *F. graminearum* at 36 Mb *and F. verticillioides* at 42 Mb (Ma *et al.*, 2010; Thatcher *et al.*, 2012). The larger genome size of *F. oxysporum* can be attributed in part to genome duplications and horizontal acquisition of supernumerary lineage-specific (LS) chromosomes or chromosome regions (2016b; Ma *et al.*, 2010; van Dam *et al.*, 2017; Vlaardingerbroek *et al.*, 2016). In *Fol*, the LS regions are rich in repetitive elements and contain genes that encode known or putative effector proteins (Ma *et al.*, 2010). Among them, 14 genes were identified that encode small, cysteine-rich proteins, known as SIX (secreted in xylem) proteins, secreted into the xylem during host infection (Houterman *et al.*, 2007; Schmidt *et al.*, 2013). A number of SIX proteins (SIX1, SIX3, SIX5 and SIX6) have been associated with host specificity as virulence determinants, i.e. effectors (Gawehns *et al.*, 2014; Lievens *et al.*, 2009; Ma *et al.*, 2015; Rep *et al.*, 2005), and three (SIX1, SIX3 and SIX4) behave as avirulence determinants when corresponding resistance genes are present in tomato (Houterman *et al.*, 2008, 2009 ; Rep *et al.*, 2004).

Several genes for resistance to *Fol* (*I*, *I-2*, *I-3* and *I-7*) have been isolated (Catanzariti *et al.*, 2015, 2017; Gonzalez-Cendales *et al.*, 2016; Ori *et al.*, 1997; Simons *et al.*, 1998). *I* and *I-7* encode leucine-rich repeat receptor proteins (LRR RPs), *I-2* encodes a coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) protein and *I-3* encodes an S-receptor-like kinase (SRLK). The SIX3 (Avr2) protein is translocated into the plant cell and recognized intracellularly by I-2 (Houterman *et al.*, 2009; Ma *et al.*, 2013), whereas SIX1 (Avr3) and SIX4 (Avr1) are recognized in the apoplast by the plasma membrane-anchored I-3 and I RPs, respectively (Catanzariti *et al.*, 2015, 2017). The effector recognized by I-7 has not yet been identified, but is likely to be a SIX protein.

Homologues of *Fol SIX* genes have been identified in several other formae speciales of *F. oxysporu*m (Laurence *et al.*, 2015; Li *et al.*, 2016; Meldrum *et al.*, 2012; Niño-Sánchez *et al.*, 2015; Rocha *et al.*, 2016; Schmidt *et al.*, 2016; Taylor *et al.*, 2016; Thatcher *et al.*, 2012; van Dam and Rep, 2017; van Dam *et al.*, 2017; Williams *et al.*, 2016). A common feature of the genomic location of several *SIX* genes is the presence of miniature impala (mimp) transposons upstream of the coding region. This feature was used to identify 11 candidate effector genes in the genome sequence of *F. oxysporum* f. sp*. melonis* (*Fom*). Eight of the 11 had homologues in other formae speciales, whereas three were unique to *Fom*. One of these was identified as the *AVRFOM2* gene encoding the effector recognized by the FOM2 resistance protein (Schmidt *et al.*, 2016). In legume-infecting formae speciales of *F. oxysporum*, mimps were identified upstream of homologues of *SIX1*, *SIX4*, *SIX8*, *SIX9*, *SIX13* and *SIX14* (Williams *et al.*, 2016). However, in this case, the initial effector identification strategy was based on the prediction of small secreted proteins from RNA sequencing (RNAseq) assemblies of host-infected tissue. Similarly, homologues of *SIX10* and *SIX12* have been found in the onion-infecting f. sp. *cepae*, and homologues of *SIX7* and *SIX10* have been found in the date palm-infecting f. sp. *canariensis* (Laurence *et al.*, 2015; Taylor *et al.*, 2016). However, to date, the role of these *SIX* homologues in pathogenicity has not been established for these formae speciales.

Functional analyses of *SIX1*, *SIX4* and *SIX6* homologues present in Brassicaceae- and Cucurbitaceae-infecting formae speciales have shown a role in host infection. Gene deletion mutants of the *SIX1* homologue in the cabbage-infecting f. sp. *conglutinans* and the *SIX4* homologue in the *Arabidopsis*-infecting strain *Fo5176* showed reduced virulence on their susceptible hosts plants, suggesting a role in pathogenicity (Li *et al.*, 2016; Thatcher *et al.*, 2012). Gene deletion of the *SIX6* homologue in the watermelon-infecting f. sp. *niveum* (*Fon*) significantly enhanced virulence on a resistant watermelon cultivar, suggesting that *SIX6* may act as an avirulence determinant, i.e. an effector recognized by a melon R protein. Moreover, transformation of a highly virulent strain of *Fon* lacking *SIX6* with *Fon SIX6* resulted in reduced virulence on otherwise susceptible watermelon plants, confirming that *SIX6* is an avirulence gene in the watermelon–*Fon* pathosystem (Niu *et al.*, 2016).

In this study, RNAseq data from roots and stems of cape gooseberry plants infected with a highly virulent strain of *Foph* were searched for homologues of *F. oxysporum* effector genes expressed during infection. RNAseq reads were mapped against the *Fol* 4287 LS transcriptome and an *F. oxysporum* effector database, and homologues of several *SIX* genes were identified. Two homologues of the *SIX1* gene were tested for their ability to complement virulence lost on susceptible tomato plants and avirulence lost on resistant (*I-3*) tomato plants by a *SIX1* knockout mutant of *Fol.*

RESULTS

Highly conserved *Fol* effectors in *Foph*

Homologues of *SIX1*, *SIX7*, *SIX10*, *SIX12* and *SIX15* (Genbank accession KY073750) were identified and their transcript sequences obtained by mapping *Foph* RNAseq reads against the *Fol* LS transcriptome (Tables 1 and S1, see Supporting Information). A homologue of FoAve1 (a *Fol* homologue of the *Verticillium dahliae* effector *Ave1*) was also identified. The presence of the *SIX1*, *SIX7*, *SIX10*, *SIX12* and *Ave1* homologues was confirmed by polymerase chain reaction (PCR) amplification from *Foph* genomic DNA (using the primers shown in Table S2, see Supporting Information) and sequencing, which also enabled full-length coding sequences to be obtained for *SIX1*, *SIX7*, *SIX10* and *SIX12*. The putative effector gene FOXM_16306 from legume-infecting formae speciales (Williams *et al.*, 2016) was the only effector transcript mapped by *Foph* RNAseq reads that did not have a homologue in *Fol.*

Table. 1 *Fusarium oxysporum* effector genes or candidate effector genes with homologues expressed in *Fusarium oxysporum* f. sp. *physali* (*Foph*)-infected cape gooseberry at 4 days post-infection.

Transcript ID	Protein product	Nucleotide position on *Fol*-4287 chromosome 14	Size (amino acids)		Protein identity (%)
			Fol	*Foph*	
FOXG_16418T0*	SIX1	1282269-1283123	284	284, 283§	72, 80§
MG647014†	SIX7	1096837-1097467	163	163	99
FOXG_17457T0*	SIX10	1092281-1092800	149	149	99
KU710369†	SIX12	1094024-1094455	127	127	100
KY073750†	SIX15	1160235-1160458	79	79	96
JQ283440†	Ave1	1366759-1367964	125	124**	89
FOXM_16306		f. sp. *medicaginis*	130‡	130**	95

†Genbank ID.

‡Predicted size of the *Fusarium oxysporum* f. sp. *melonis* (*Fom*) protein reported by Williams *et al.* (2016).

§Two *SIX1* homologues were identified in *Foph*.

*EnsemblFungi ID.

**Expected size based on similarity to the corresponding *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) or f. sp. *medicaginis* (*Fome*) protein.

The predicted protein sequences encoded by these *Foph* homologues were aligned with their *Fol* and *F. oxysporum* f. sp. *medicaginis* (in the case of FOXM_16306) counterparts using ClustalW (Fig. S1, see Supporting Information). *Foph* SIX7, SIX10, SIX12 and SIX15 showed 96%–100% protein identity with their *Fol* homologues, whereas *Foph* Ave1 only showed 87% identity (Table 1). FOXM_16306 showed 95% identity to its *Foph* counterpart.

Phylogenetic analysis of these sequences was performed using homologues identified by blastp searches of the National Center for Biotechnology Information (NCBI) protein database and tblastn searches of the nucleotide and whole-genome shotgun contig databases. The phylogenetic trees based on SIX7, SIX10, SIX12, SIX15 and Ave1 protein sequences each showed that the *Foph* sequences were more closely related to their *Fol* homologues than to homologues from other formae speciales (Fig. S2, see Supporting Information). Interestingly, the *Fusarium oxysporum* homologues of FOXM_16306 formed a distinct clade embedded in a more ancient lineage of *Colletotrichum* sequences (Fig. S2), suggesting that FOXM_16306 could be present in *F. oxysporum* as a consequence of lateral gene transfer from *Colletotrichum.*

Evidence for a segment of *Fol* chromosome 14 conserved between *Fol* and *Foph*

Given that the *Foph SIX7*, *SIX10* and *SIX12* genes are located in a 5.2-kb region on *Fol* chromosome 14 (Schmidt *et al.*, 2013; Fig. 1) and show very high nucleotide identity (99.0%, 99.6% and 100%, respectively) with their *Fol* counterparts (Fig. S3, see Supporting Information), it is possible that this region has undergone a relatively recent lateral transfer between *Foph* and *Fol.* To test whether the entire region containing this gene cluster was present in *Foph*, two pairs of primers (Inter1F/R and Inter2F/R) were designed to amplify the intergenic regions between *SIX10* and *SIX12* (Int-1) and between *SIX12* and *SIX7* (Int-2) (Fig. 1, Table S2). The Int-1 and Int-2 PCR products were similar in size to the corresponding *Fol* intergenic regions, and sequencing confirmed their high sequence identity (Fig. S4, see Supporting Information). Excluding the 5′ end of the *SIX10* coding sequence, *Foph* Int-1 only differs from *Fol* Int-1 by an indel of 214 bp, which corresponds to a mimp2 element, and two nucleotide changes in 1.417 kb (99.9% identity). Similarly, excluding the 5′ ends of the *SIX7* and *SIX12* coding sequences, *Foph* Int-2 only differs from *Fol* Int-2 by two indels, and two single nucleotide deletions and three nucleotide changes in 1.232 kb (99.6% identity) (Fig. S4). Overall, these data suggest the presence of a SIX gene cluster in *Foph* nearly identical to that in *Fol* (Fig. 1), with intergenic regions modified by the differential movement of transposable elements. Moreover, the similarity between the *Fol* and *Foph SIX7/SIX10/SIX12* gene clusters is significantly greater ($\chi^2 = 12.54$, $P < 0.001$) than that for at least one gene in the core genome, *FEM1*, which shows 98.6% nucleotide identity between *Foph* and *Fol* (Fig. S5, see Supporting Information).

The *SIX15* gene is located close to this cluster and, like *SIX7, SIX10* and *SIX12*, shows high similarity (98.8% identity) to its *Fol* counterpart (Figs 1 and S3), raising the possibility that this region of high conservation with *Fol* chromosome 14 extends to and includes *SIX15*. Four annotated genes lie between SIX7 and SIX15. *Foph* RNAseq reads mapped to FOXG_17458 and FOXG_17460, but no reads mapped to FOXG_17459 or FOXG_17461. However, blastn searches of the *Fol* genome sequence showed that the reads mapping to FOXG_17458, which encodes an FTF1 transcription factor, and FOXG_17460 showed higher matches to FOXG_17123 on LS chromosome 6 and FOXG_17180, respectively. Thus, no

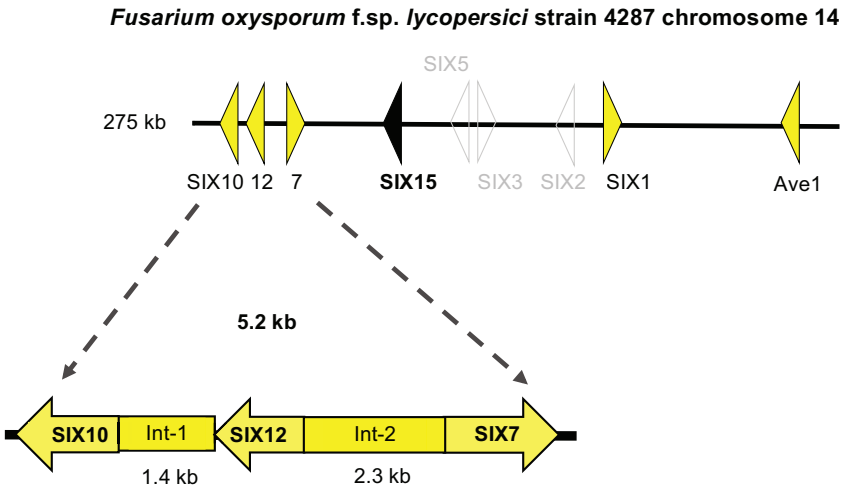

Fig. 1 Schematic representation of the 275-kb region of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) chromosome 14 containing the homologues shared between *Fol* and *Fusarium oxysporum* f. sp. *physali* (*Foph*). The presence of genes and intergenic regions shown in yellow was confirmed by amplification from *Foph* genomic DNA and sequencing. The *SIX15* gene shown in black was identified and its coding sequence obtained by mapping *Foph* RNA sequencing (RNAseq) reads to the *Fol SIX15* coding sequence (GenBank accession KY073750). Effector genes *SIX2*, *SIX3* and *SIX5* shown in grey and located between *SIX15* and *SIX1* were not identified in *Foph*. An expansion of the *SIX10–SIX12–SIX7* gene cluster shows that the intergenic regions Int-1 and Int-2 are also conserved between *Fol* and *Foph*. [Colour figure can be viewed at wileyonlinelibrary.com]

evidence was found for lateral transfer of a larger gene cluster than the *SIX7*/*SIX10*/*SIX12* cluster alone.

Two *SIX1* homologues are present in *Foph*

Two homologues of *Fol SIX1*, designated *SIX1a* and *SIX1b*, were detected during RNAseq mapping and were confirmed by the presence of mixed peaks in the sequence chromatograms of the *Foph SIX1* PCR product (Fig. S6, see Supporting Information). To determine the correct sequence of each *SIX1* gene in *Foph*, two primer pairs SIX1.1F/R and SIX1.2F/R (Table S2) were designed based on the sequence for each homologue inferred from the RNAseq mapping. These primers were used to amplify, clone and sequence the individual *SIX1* homologues from *Foph*. The predicted protein sequences encoded by the *SIX1a* and *SIX1b* genes were aligned with their counterparts from *Fol* using ClustalW, and were found to have 72% and 80% identity to *Fol* SIX1, respectively (Table 1, Fig. S7, see Supporting Information). The predicted protein sequences of *Foph SIX1a* and *SIX1b* were used to identify SIX1 homologues from other formae speciales using BLASTP searches of the NCBI protein databases and TBLASTN searches of the nucleotide and whole-genome shotgun contig databases. Protein alignment and Bayesian phylogenetic analysis of the SIX1 homologues using BEAST and DensiTree (Bouckaert and Heled, 2014; Drummond *et al.*, 2012) showed that *Foph* SIX1b is more closely related to *Fol* SIX1 than to the SIX1 homologues present in other formae speciales of *F. oxysporum* (Figs 2, S8 and S9, see Supporting Information).

Foph SIX1a and SIX1b transgenes are expressed in *Fol* during plant infection, but do not complement the loss of virulence in *Fol-ΔSIX1*

Fol-susceptible tomato plants inoculated with *SIX1* knockout strains of *Fol* (*Fol-ΔSIX1*) showed reduced disease compared with plants inoculated with wild-type *Fol*, whereas tomato plants inoculated with *Fol-ΔSIX1* strains complemented with *Fol*-SIX1 (*Fol-ΔSIX1*:*Fol*-SIX1) showed restoration of disease symptoms (Rep, 2005). To test whether *Foph SIX1a* and/or *SIX1b* can restore virulence in *Fol*-ΔSIX1 tomato plants, 10 *Fol*-ΔSIX1:*SIX1a* and six *Fol*-ΔSIX1:*SIX1b* transformants were generated and confirmed by PCR amplification (Fig. S10, see Supporting Information). Tomato seedlings (M82 cultivar susceptible to *Fol* race 3) were then inoculated with *Fol*-WT, *Fol-ΔSIX1*, *Fol-ΔSIX1:SIX1a* and *ΔSIX1:SIX1b* transformants. Plants inoculated with either *Fol*-ΔSIX1:*SIX1a* or *Fol*-ΔSIX1:*SIX1b* transformants did not show restoration of disease symptoms (Figs 3, 4 and S11, see Supporting Information), with the possible exception of one *Fol*-ΔSIX1:*SIX1a* transformant (designated SIX1a.16 in Fig. 3). Given that none of the other nine *Fol*-ΔSIX1:*SIX1a* transformants showed a similar restoration of disease symptoms, this result appears to be an anomaly rather than an indication of complementation. An alternative explanation might be that transgene insertion has generated a suppressor mutation whose effect would be immediately apparent given that *Fol* is a haploid fungus. Overall, these results indicate that neither *Foph SIX1* gene complemented the loss of virulence function in *Fol*-ΔSIX1.

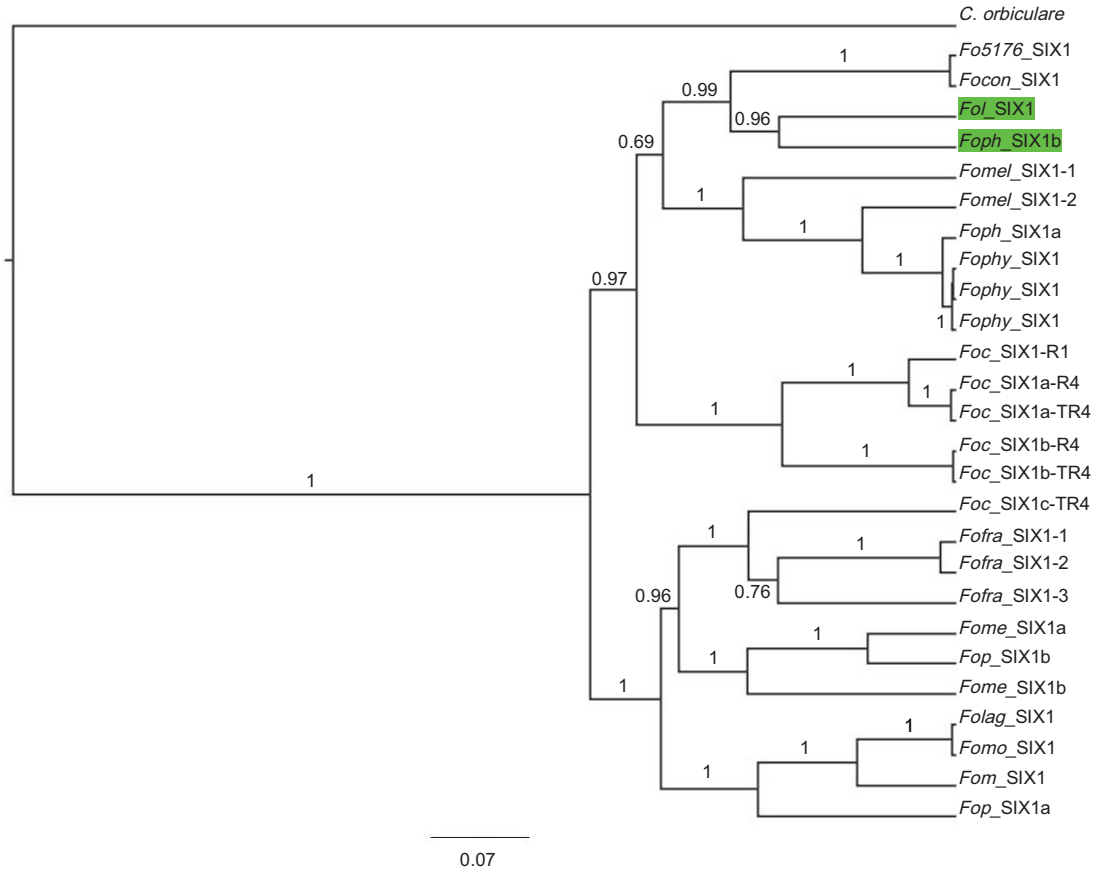

Fig. 2 Phylogenetic tree of SIX1 proteins from various formae speciales of *Fusarium oxysporum*. The phylogenetic analysis was conducted using BEAST and the protein sequence alignment shown for SIX1 in Fig. S8 (see Supporting Information). A *Colletotrichum orbiculare* homologue of SIX1 was used as an outlier. Internal node supports are indicated as Bayesian probabilities. A key to the labels used for each forma specialis is provided in Fig. S8. The scale bar indicates time in millions of years. *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) SIX1 and *Fusarium oxysporum* f. sp. *physali* (*Foph*) SIX1b are highlighted in green to show their close phylogenetic relationship. [Colour figure can be viewed at wileyonlinelibrary.com]

To test whether the SIX1a and SIX1b transgenes were expressed during infection, a reverse transcriptase-polymerase chain reaction (RT-PCR) analysis using *Foph SIX1a*, *Foph SIX1b* and *FEM1* primers (Table S2) was performed on roots of susceptible tomato plants infected with three *Fol-ΔSIX1:SIX1a* transformants and three *Fol-ΔSIX1:SIX1b* transformants (Fig. 5). These were compared with mock-inoculated plants and plants inoculated with *Fol*-WT or *Fol-ΔSIX1* at 3 and 6 days post-inoculation (dpi). Expression of *Foph SIX1a* and *Foph SIX1b* was detected in the 3- and 6-dpi samples from tomato roots inoculated with *Fol-ΔSIX1:SIX1a* and *Fol-ΔSIX1:SIX1b* transformants, respectively. No *Foph SIX1a* or *Foph SIX1b* expression was detected in root samples from *Fol*-WT, *Fol-ΔSIX1* or mock-inoculated tomato plants. These results showed that the lack of complementation was not a result of a lack of transgene transcription.

Foph SIX1b complements the loss of avirulence of *Fol-ΔSIX1* on tomato plants carrying the *I-3* resistance gene

The tomato *I-3* resistance gene confers resistance to *Fol* race 3 through recognition of the SIX1 effector (Catanzariti *et al.*, 2015; Rep *et al.*, 2004). To test whether *I-3* can also recognize *Foph SIX1a* or *SIX1b*, 11-day-old IL7-3 tomato plants were inoculated with two *Fol-ΔSIX1:SIX1a* transformants and two *Fol-ΔSIX1:SIX1b* transformants. The IL7-3 introgression line of tomato contains the *I-3* gene and is resistant to *Fol*-WT, but susceptible to *Fol-ΔSIX1*.

Tomato plants inoculated with *Fol-ΔSIX1* or the *Fol-ΔSIX1:SIX1a* transformants showed significantly more disease compared with those inoculated with *Fol*-WT (Fig. 6A). Plants inoculated with the *Fol-ΔSIX1:SIX1b* transformants showed

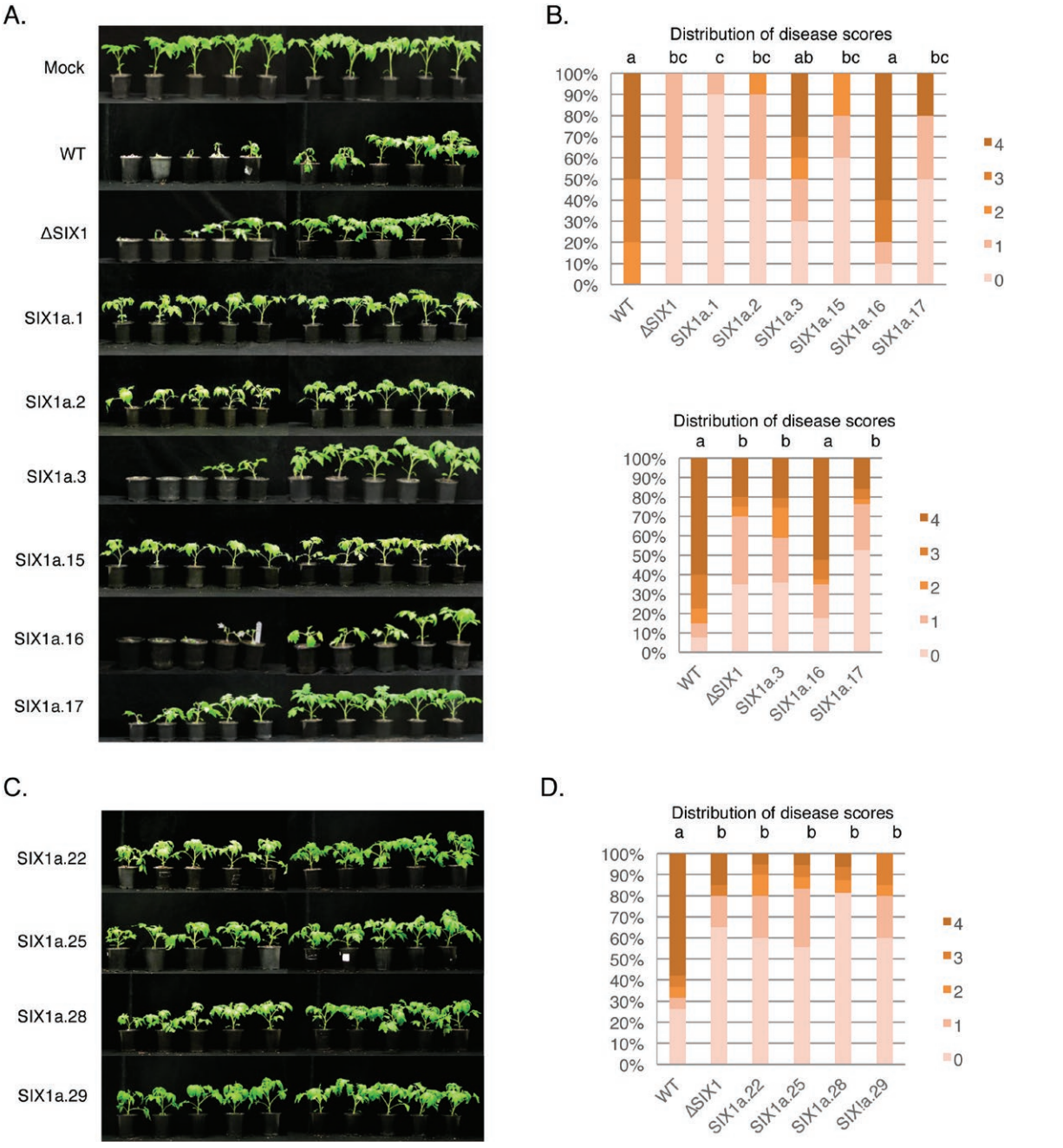

Fig. 3 Pathogenicity tests on M82 tomato plants with *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) and 10 *Fol-ΔSIX1:SIX1a* (*SIX1a*) transformants. (A, C) Photographs taken at 21 days post-inoculation (dpi) of infected M82 plants from two experiments testing all 10 transformants. (B, D) Top panel in (B) shows the distribution of disease scores for plants shown in (A). Bottom panel in (B) shows the distribution of disease scores at 21 dpi for plants infected with wild-type (WT), *ΔSIX1* or *SIX1a* transformants 3, 16 or 17 (the transformants showing the highest disease scores from A) pooled from four replicate experiments ($n = 38–40$; results of individual replicates are shown in Fig. S11, see Supporting Information). (D) shows the distribution of disease scores at 21 dpi for plants infected with WT, *ΔSIX1* or *SIX1a* transformants 22, 25, 28 or 29 pooled from two replicate experiments ($n = 18–20$; results of individual replicates are shown in Fig. S11). Treatments with different letters are significantly different at $P = 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

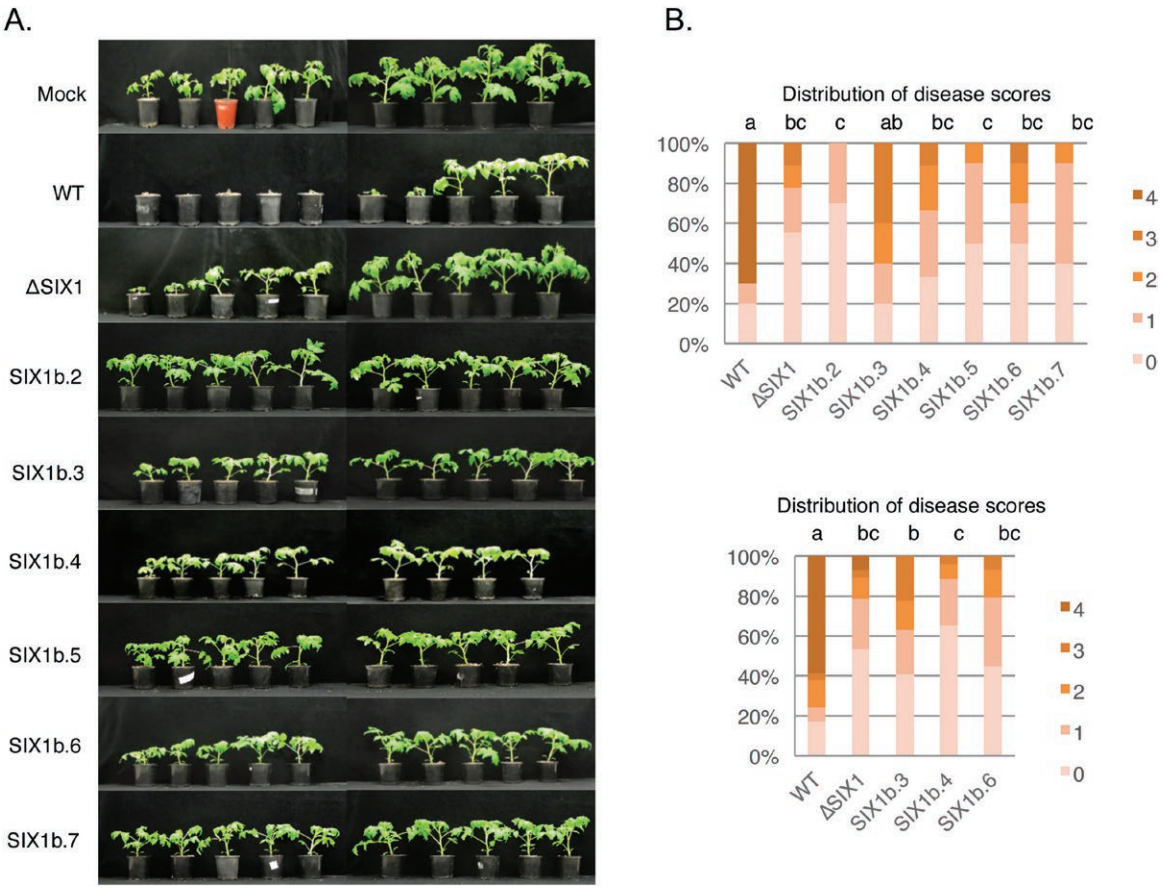

Fig. 4 Pathogenicity tests on Moneymaker tomato plants with *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) and six *Fol-ΔSIX1:SIX1b* (*SIX1b*) transformants. (A) Photographs taken at 21 days post-inoculation (dpi) of infected Moneymaker plants from an experiment testing all six transformants. (B) Top panel shows the distribution of disease scores for the plants shown in (A). Bottom panel shows the distribution of disease scores from three replicate experiments ($n = 26–29$) with wild-type (WT), *ΔSIX1* or *SIX1b* transformants 3, 4 or 6 (the transformants showing the highest disease scores from A). Treatments with different letters are significantly different at $P = 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

significantly less disease compared with those inoculated with *Fol-ΔSIX1* or the *Fol-ΔSIX1:SIX1a* transformants, and no significant difference compared with plants inoculated with *Fol*-WT (Fig. 6B), suggesting that *Foph-SIX1b* is recognized by the tomato *I-3* resistance gene.

To confirm the avirulence of the *Fol-ΔSIX1:SIX1b* transformants observed on tomato plants carrying *I-3*, all six *Fol-ΔSIX1:SIX1b* transformants generated in this study were tested for pathogenicity on IL7-3 plants (Fig. 7A). The results showed no significant difference in disease scores when plants inoculated with the six *Fol-ΔSIX1:SIX1b* transformants were compared with plants inoculated with *Fol*-WT, whereas plants inoculated with *Fol-ΔSIX1* were significantly more diseased (Fig. 7B). These results corroborate the previous finding, suggesting that *Foph* SIX1b is recognized as an avirulence factor by the I-3 resistance protein.

Fol and *Foph SIX1* sequences show evidence for both diversifying and purifying selection

Some of the 36 residues conserved between the mature *Fol* SIX1 and *Foph* SIX1b proteins, but not SIX1a (Fig. 8), are presumably important for the recognition of *Fol* SIX1 and *Foph* SIX1b by I-3, whereas some of the 42 residues unique to the mature *Fol* SIX1 protein (relative to *Foph* SIX1a and SIX1b) are presumably important for pathogenic function in tomato. An analysis of SIX1 sequences across 26 formae speciales of *F. oxysporum* revealed a hypervariable region (corresponding to V130–T184 in *Fol* SIX1; Fig. S8), with more than half the positions (26/42 residues excluding four conserved cysteines) showing evidence for diversifying selection (Fig. 8). In contrast, the signal peptide, pro-peptide and relatively conserved C-terminal (from F185 in *Fol* SIX1) regions contain the majority of the positions (30/38 residues) showing evidence for purifying selection (Fig. 8). Surprisingly, the hypervariable region also contains almost half of the residues

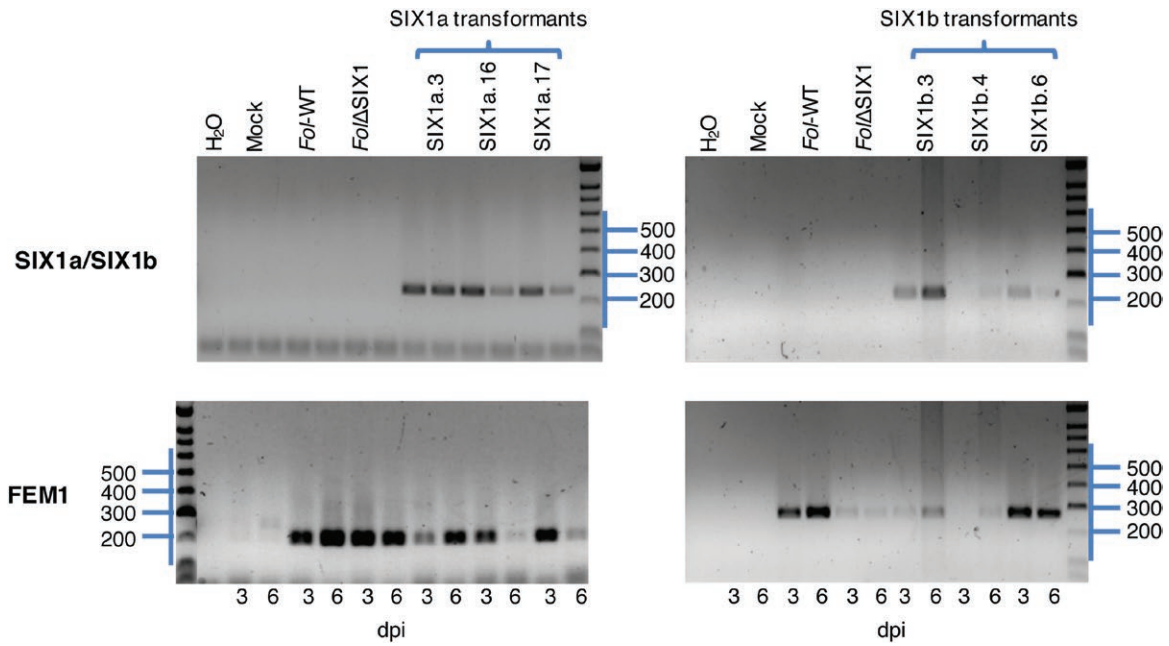

Fig. 5 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showing the expression of *Fusarium oxysporum* f. sp. *physali* (*Foph*) *SIX1a* or *Foph SIX1b* transgenes in tomato roots infected with *Fol-⊿SIX1:SIX1a/b* transformants at 3 and 6 days post-inoculation (dpi). Top gel images show bands (expected size of 250 bp) consistent with *SIX1a* and *SIX1b* expression in *Fol-⊿SIX1:SIX1a/b*-infected roots, compared with mock-, *Fol*-WT- or *Fol-⊿SIX1*-inoculated controls. Bottom gel images show bands (expected size of 201 bp with RT_Fem1 primers in the SIX1a experiment and 250 bp with q_Fem1 primers in the SIX1b experiment) consistent with *FEM1* expression in *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*)-infected tomato roots. [Colour figure can be viewed at wileyonlinelibrary.com]

conserved between the mature *Fol* SIX1 and *Foph* SIX1b proteins, but not SIX1a (17/36 residues), with the majority (11/17 residues) occurring at positions showing diversifying selection (Fig. 8).

Diversifying selection in effector proteins is often associated with avoidance of recognition by host resistance proteins. Although *Fol SIX1* may have evolved to avoid recognition by tomato and *Foph SIX1b* to avoid recognition by cape gooseberry, they have not evolved to avoid recognition conferred by the *I-3* gene derived from *Solanum pennellii*. Thus, it is likely that residues shared between *Fol* SIX1 and *Foph* SIX1b in the hypervariable region, particularly those at positions showing evidence for diversifying selection, will be involved in recognition. However, it is also possible that pathogen effector diversification could be necessitated by host diversification of an effector target.

The hypervariable region also contains a high proportion of the residues unique to the mature *Fol* SIX1 protein relative to *Foph* SIX1a and SIX1b (19/42), with the majority of these residues (11/19) also occurring at positions showing evidence for diversifying selection (Fig. 8). However, the majority of the remaining residues unique to the mature *Fol* SIX1 protein (22/42) occur in the relatively conserved C-terminal region (from F185 onwards in *Fol* SIX1), almost half (9/22) of which are in positions showing evidence of diversifying selection (Fig. 8). Although residues unique to the mature *Fol* SIX1 protein may contribute to pathogenicity, a role in recognition cannot be excluded for positions in which SIX1b also contains a unique residue. Interestingly, the majority (17/19) of the positions in the hypervariable region with residues unique to *Fol* SIX1 also have unique residues in SIX1b, whereas the majority (14/22) of the positions in the C-terminal region with residues unique to *Fol* SIX1 have residues shared in common between *Foph* SIX1b and SIX1a.

DISCUSSION

Cape gooseberry (*Physalis peruviana*) is an economically important crop plant in Colombia that is under threat from vascular wilt disease caused by *Foph*. Little is known about the interaction between *Foph* and its host plant. In this study, seven putative effector transcripts were identified by mapping of RNAseq data from *Foph*-infected cape gooseberry plants against the *Fol* LS transcriptome and a database of putative effectors identified in other formae speciales of *F. oxysporum*. Six were homologues of the *Fol* genes *SIX1*, *SIX7*, *SIX10*, *SIX12, SIX15* and *Ave1*. The seventh was a homologue of the candidate effector FOXM_16306 from legume-infecting formae speciales of *F. oxysporum* (Williams *et al.*, 2016).

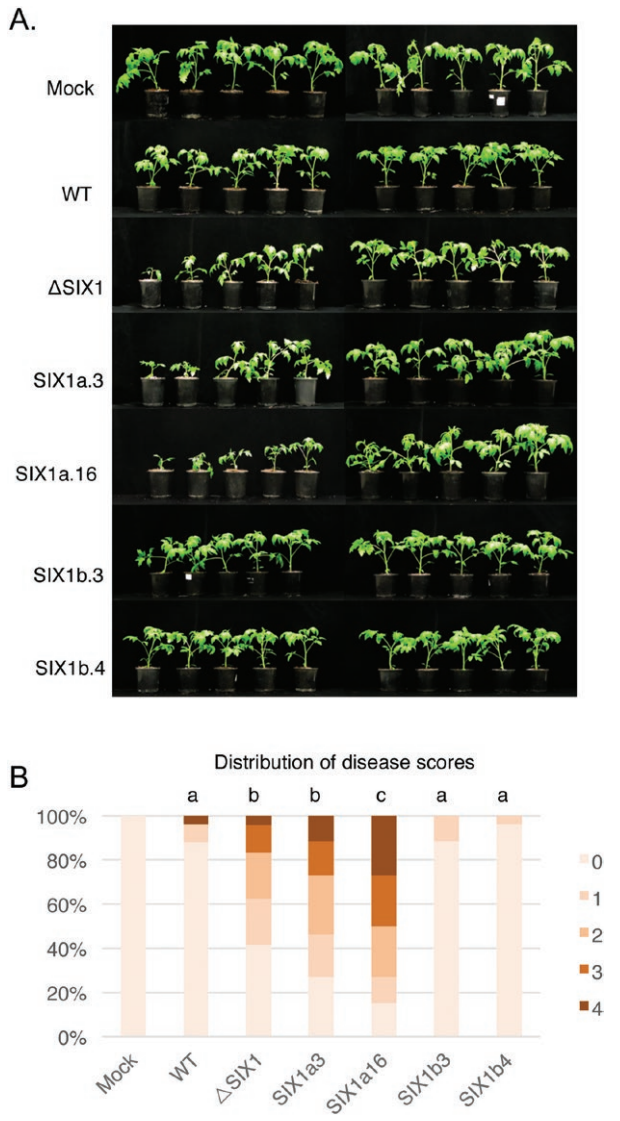

Fig. 6 Pathogenicity tests on IL7-3 tomato plants with *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) or *Fol-ΔSIX1:SIX1a/b* transformants. (A) Photographs taken at 21 days post-inoculation (dpi) of IL7-3 plants infected with Fol-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*), *Fol-ΔSIX1:SIX1a* transformants 3 or 16 or *Fol-ΔSIX1:SIX1b* transformants 3 or 4 from one of three replicate experiments. (B) Distribution of disease scores at 21 dpi for plants shown in (A) and two additional replicates ($n = 28–30$). Treatments with different letters are significantly different at $P = 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

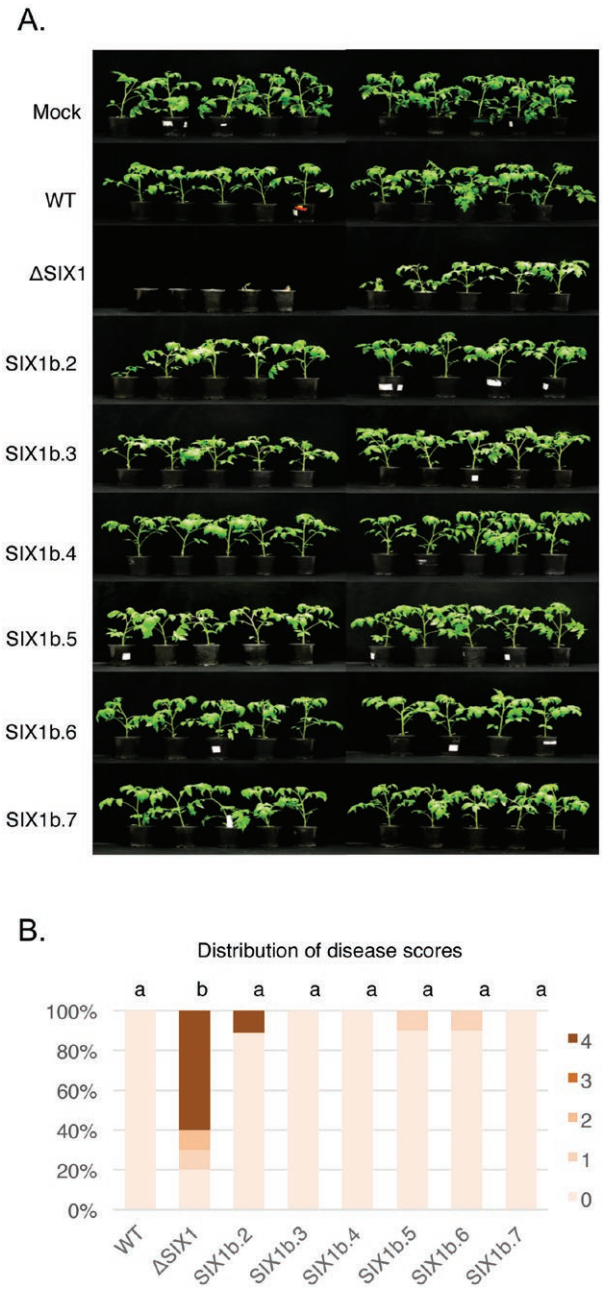

Fig. 7 Pathogenicity tests on IL7-3 tomato plants with *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) or all six *Fol-ΔSIX1:SIX1b* transformants. (A) Photographs taken at 21 days post-inoculation (dpi) of IL7-3 plants infected with *Fol*-WT (WT), *Fol-ΔSIX1* (*ΔSIX1*) or *Fol-ΔSIX1:SIX1b* transformants 2–7. (B) Distribution of disease scores for the plants shown in (A) ($n = 9–10$). Treatments with different letters are significantly different at $P = 0.05$.

Lateral transfer of a *SIX7*/*SIX10*/*SIX12* gene cluster

The identification of a highly conserved cluster of physically linked genes (*SIX7*, *SIX10* and *SIX12*) shared between *Foph* and *Fol* suggests a relatively recent lateral transfer of a segment of *Fol* chromosome 14 between *Fol* and *Foph*. In most formae speciales that have been sequenced to date, *SIX7*, *SIX10* and *SIX12* are either all present (e.g. ff. spp. *lycopersici*, *dianthi*, *gladioli*, *narcissi* and *zingiberi*) or all absent. Similarly, *SIX7*, *SIX10* and *SIX12* have been shown to occur together in formae speciales that have been surveyed for their presence by PCR (e.g. ff. spp. *cepae* and *lini*). This pattern of presence/absence could suggest their transmission throughout *F. oxysporum* as a unit, and indeed a search of

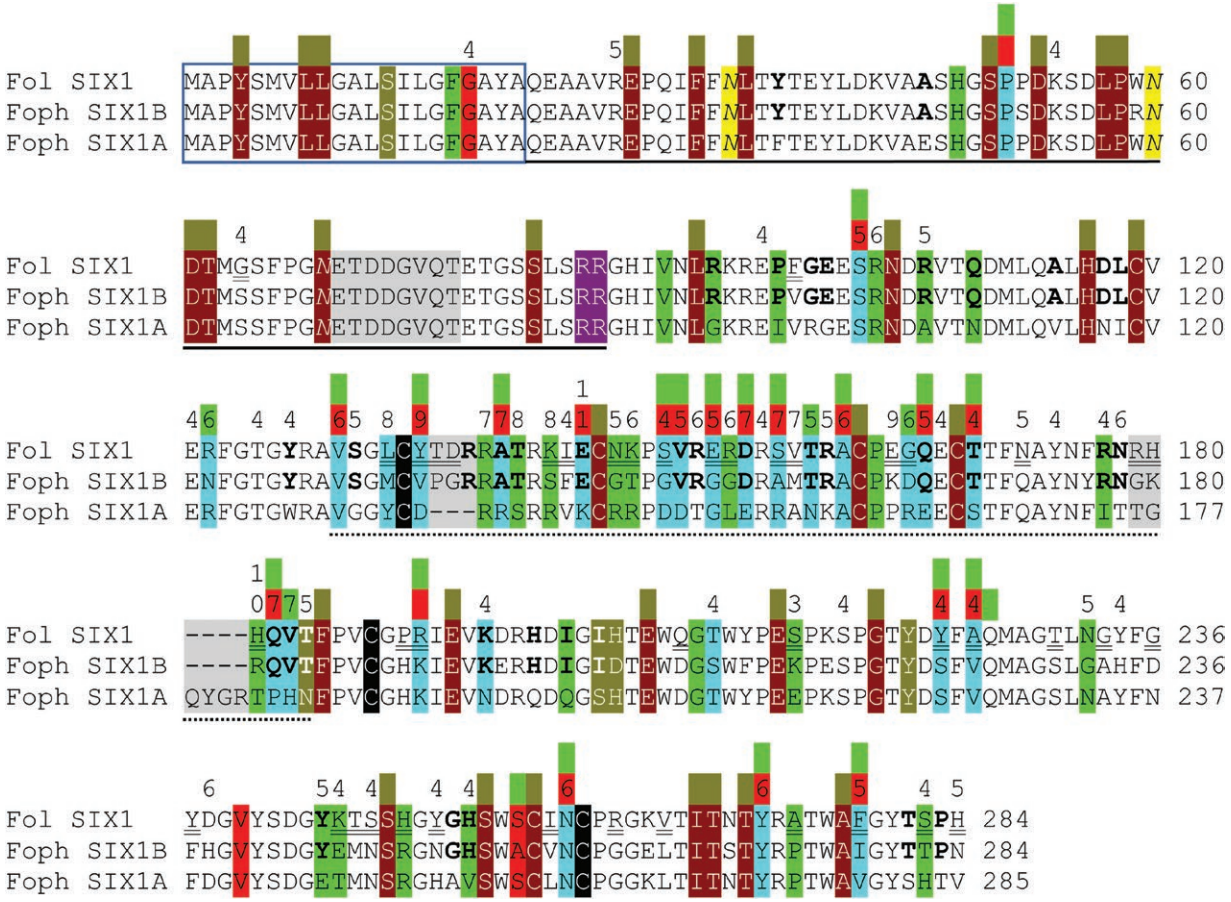

Fig. 8 Sequence alignment of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) SIX1, *Fusarium oxysporum* f. sp. *physali* (*Foph*) SIX1a and *Foph* SIX1b highlighting positions showing evidence for diversifying and purifying selection among 18 different SIX1 sequences from *Fusarium oxysporum*. Positions showing evidence for diversifying selection detected using FUBAR, FEL or MEME are highlighted in blue, red and green, respectively. Positions showing evidence for purifying selection detected using FUBAR or FEL are highlighted in dark brown and olive green, respectively. Numbers above the sequence alignment indicate hypervariable positions showing four or more different residues among the 18 sequences analysed. Regions highlighted in grey indicate positions excluded from analysis owing to deletions of three or more amino acid residues in a number of sequences. The predicted signal peptide is boxed and the predicted pro-peptide region delimited by a Kex2 cleavage site (highlighted in purple) is underlined. Asparagine residues in predicted *N*-glycosylation sites are shown in italics and are highlighted in yellow unless located at a position showing purifying selection. Cysteine residues predicted to be involved in disulfide bond formation are highlighted in black unless located at a position showing purifying selection. A hypervariable region showing a high proportion of positions undergoing diversifying selection is underlined with a dotted line. Residues shared between *Fol* SIX1 and *Foph* SIX1b are shown in bold font. Residues unique to *Fol* SIX1 (relative to *Foph* SIX1a and SIX1b) are double underlined. [Colour figure can be viewed at wileyonlinelibrary.com]

the NCBI genome shotgun sequence database confirms the presence of a 3.46-kb *SIX7*/*SIX10*/*SIX12* cluster in f. sp. *gladioli* and a 4.31-kb cluster in f. sp. *narcissi*. Given that chromosome 14 is rich in transposable elements, it is possible that such a unit could be transmitted as a macro-transposon.

Clustering of these genes might also reflect a cooperative function similar to that observed for *SIX3* (*Avr2*) and *SIX5* (Ma *et al*., 2015). However, the presence of *SIX7*, but the absence of *SIX10* and *SIX12*, in ff. spp. *lilii* and *cubense* (Czislowski *et al*., 2017; van Dam *et al*., 2017) suggests that *SIX7* may be separable, both physically and functionally, from *SIX10* and *SIX12*. Moreover, *SIX10* and *SIX12* are not arranged in divergent orientation, and so cannot be transcribed coordinately from a shared promoter, as is the case for SIX3 and SIX5. Attempts to knock out *SIX7*, *SIX10* and *SIX12*, either as a group or individually, have so far failed, but will be essential to understand their role in pathogenicity.

Additional *Foph* strains also need to be analysed for the presence of this segment of chromosome 14 to determine whether it is a general feature in *Foph* or unique to this particular isolate. The *SIX7*/*SIX10*/*SIX12* cluster of genes is absent from three accessions of *Fusarium oxysporum* f. sp. *physalis* (*Fophy*) isolated from an unspecified species of *Physalis* in California (van Dam *et al*., 2017), presumably *P. philadelphica* (syn. *P. ixocarpa*), the tomatillo, which is widely grown in California. Conversely,

SIX15, which is present in *Fophy*, shows no relationship with the presence/absence of *SIX7*, *SIX10* and *SIX12* in other formae speciales of *F. oxysporum*, suggesting that any physical association with *SIX7*, *SIX10* and *SIX12* is easily disrupted. No evidence for a physical association between *Foph SIX15* and the *Foph SIX7*/*SIX10*/*SIX12* gene cluster was found in this study, but it cannot be excluded. The generation of an assembled *Foph* genome sequence will be required to examine this possibility.

Complementation of avirulence, but not virulence, separates SIX1 recognition from its role in pathogenicity

The presence of less conserved homologues of *Fol SIX1* and *Ave1*, which are also located on *Fol* chromosome 14, suggests that these effector genes may have a different ancestry from *SIX7*, *SIX10*, *SIX12* and *SIX15*, perhaps via the acquisition of different segments of the pathogenicity chromosome at different times in the evolution of *Fol* and *Foph*. Homologues of the *SIX1* gene have been identified in other formae speciales of *F. oxysporum* (Laurence *et al.*, 2015; Meldrum *et al.*, 2012; Niño-Sánchez *et al.*, 2015; Rocha *et al.*, 2016; Schmidt *et al.*, 2016; Thatcher *et al.*, 2012; Williams *et al.*, 2016; Fig. S8). They share highly similar signal peptide and prodomain sequences, but show highly diverse mature protein sequences with some amino acid positions showing considerable variation (Fig. S8), including positions whose corresponding codon sequences show evidence for diversifying selection (Fig. 8).

In *Fol*, the *SIX1* effector is required for full pathogenicity (Rep *et al.*, 2002, 2004) and its expression during penetration of the root cortex suggests that *SIX1* may play a role in the biotrophic stage of *Fol* infection (van der Does *et al.*, 2008). Functional investigation of the *SIX1* homologue present in f. sp. *conglutinans* has shown that *SIX1* also plays a role in host virulence in cabbage (Li *et al.*, 2016). However, *Fol SIX1* was unable to complement a loss of endogenous *SIX1* function in f. sp. *conglutinans* (Li *et al.*, 2016). Similarly, in this study, neither *SIX1a* nor *SIX1b* could complement the loss of *SIX1* pathogenicity in a *Fol-*$\triangle$*SIX1* knockout mutant, suggesting that *SIX1a* and *SIX1b* may play a specific role in *Foph* pathogenicity. Overall, these results support the suggestion that *F. oxysporum SIX1* effectors are associated with host specificity (Li *et al.*, 2016).

Fol SIX1 has also been characterized as an avirulence gene (*Avr3*), as its protein product is recognized by the I-3 protein, which confers resistance to *Fol* races 2 and 3 as a consequence (Catanzariti *et al.*, 2015; Rep *et al.*, 2004, 2005). Although *SIX1a* and *SIX1b* do not complement the pathogenicity function of *Fol SIX1*, this study showed that SIX1b is able to trigger resistance to *Fol* race 3 in tomato plants carrying *I-3*, and is therefore recognized by the I-3 protein (Figs 6B and 7A). This finding separates the recognition of SIX1 from its role as an effector, i.e. it shows that a pathogenic effect in tomato is not required for recognition.

Overall, the compositional differences between *Fol* SIX1, *Foph* SIX1a and *Foph* SIX1b point to an important role for residues in the hypervariable region of SIX1 in recognition by I-3, and perhaps a role for residues in the C-terminal region in host specificity/pathogenicity. Future studies of these residues by mutation or domain swap analyses need to be carried out to further investigate the specificity of SIX1 recognition. Information on recognition specificity might also be obtained by testing for complementation of avirulence in the *Fol-*$\triangle$*SIX1* knockout by the f. sp. *conglutinans* homologue of *SIX1*, which encodes the SIX1 protein next most closely related to *Fol* SIX1 after *Foph* SIX1b (Fig. 2).

Host recognition of *Fol* and *Foph* effectors

Several genes for resistance to *Fol* (*I*, *I-2*, *I-3* and *I-7*) have been identified in tomato (Catanzariti *et al.*, 2017; Gonzalez-Cendales *et al.*, 2016; Ori *et al.*, 1997; Simons *et al.*, 1998). *I*, *I-2* and *I-3* encode proteins able to recognize specific SIX proteins. SIX1 is recognized at the plasma membrane by the I-3 SRLK, which confers resistance to *Fol* races 2 and 3 (Catanzariti *et al.*, 2015). SIX3 (Avr2) is recognized intracellularly by the I-2 CC-NB-LRR protein, which confers resistance to *Fol* race 2 (Houterman *et al.*, 2009; Ma *et al.*, 2015). SIX4 (Avr1) is recognized at the plasma membrane by the I LRR-RP, which confers resistance to *Fol* race 1 (Catanzariti *et al.*, 2017).

In melon, the AvrFom2 effector protein from *Fom* race 2 is recognized by an NB-LRR protein encoded by the *Fom-2* gene (Joobeur *et al.*, 2004), suggesting that AvrFom2 is translocated into the cytoplasm, as occurs with the SIX3 (Avr2) effector of *Fol*. The *Fom-1* gene, which confers resistance to *Fom* races 0 and 2, encodes a Toll/Interleukin-1 receptor (TIR) NB-LRR protein (Brotman *et al.*, 2013), suggesting that AvrFom1 is also translocated into the cytoplasm. In Arabidopsis, six genes (*RFO1–6*) have been identified that confer quantitative resistance to *F. oxysporum* f. sp. *matthioli. RFO1*, *2* and *3* encode a wall-associated kinase (WAK) protein, an LRR-RP and an SRLK, respectively (Cole and Diener, 2013; Shen and Diener, 2013), with the last two proteins similar to those encoded by the tomato *I*, *I-3* and *I-7* genes in tomato.

In cape gooseberry, a gene that encodes a CC-NB-LRR protein was found to be highly correlated with *Foph* resistance (Enciso-Rodríguez *et al.*, 2013). However, the identification of at least five different types of receptor protein (LRR RPs, SRLKs, WAKs, TIR-NB-LRRs and CC-NB-LRRs), including three different types of membrane receptor, able to trigger resistance against *F. oxysporum* in different host plants suggests that effector recognition in the apoplast by membrane-anchored receptors is a common defence mechanism against *F. oxysporum*. The functional evidence

obtained in this study, showing recognition of *Foph* SIX1b by the tomato I-3 resistance protein, suggests that SIX1b has the potential to be recognized by an SRLK protein in cape gooseberry. A search of cape gooseberry germplasm for different homologues of tomato I-3 might enable the identification of varieties resistant to *Foph* isolates carrying *SIX1*. Alternatively, cape gooseberry plants could be transformed with the tomato *I-3* gene to see whether it can recognize SIX1b and mediate resistance to *Foph* in cape gooseberry, thereby providing plant breeding programmes with an alternative source of resistance for the development of new cape gooseberry cultivars. However, the same strategy is unlikely to be successful in tomatillo, given that the *Fophy* homologue of *Fol* SIX1 is closely related to *Foph* SIX1a (Fig. 2), which is not recognized by *I-3* (Fig. 6).

EXPERIMENTAL PROCEDURES

RNAseq analysis

Total RNA was extracted from pooled root and stem tissue of two susceptible cape gooseberry seedlings [accession number 09U274-1 from the *in vitro* germplasm bank at the Colombian Corporation for Agricultural Research (CORPOICA), Bogotá, Colombia] at 4 dpi with *Foph* (Isolate code MAP5 from the micro-organisms germplasm bank of CORPOICA). About 10 μg of purified total RNA was used to carry out a modified SMART™ cDNA synthesis (Clontech, California, USA). Four RNAseq libraries were generated from purified cDNA and sequenced (100-bp single-end reads) on an Illumina HiSeq 2000 platform. A total of 38 874 746 RNAseq reads was obtained from the two infected cape gooseberry seedlings. The RNAseq data were generated by Carolina Gonzalez from CORPOICA, Colombia and shared as part of a collaboration established between the Disease Resistance Laboratory at the Australian National University (ANU) and the Phytosanitary Management Division at CORPOICA.

Predicted transcript sequences for genes located in the LS regions (i.e. chromosomes 3, 6, 14, 15 and segments of chromosomes 1 and 2) of *Fol* were retrieved from the *Fol* 4287 coding sequence (CDS) database at EnsemblFungi (https://fungi.ensembl.org/Fusarium_oxysporum/Info/Index; Kersey *et al.*, 2016). An additional database of 89 candidate *F. oxysporum* effectors was obtained from van Dam *et al.* (2016). Two additional candidate effectors, CRX1 and CRX2 (GenBank accessions KP965011.1 and KP965012.1), found in f. sp. *cepae* (Taylor *et al.*, 2016), and three, FOXM_15788, FOXM_109214 and FOXM_16306, found in the legume-infecting ff. spp. *medicaginis*, *ciceris* and *pisi* (Williams *et al.*, 2016), were added to the van Dam *et al.* (2016) database of putative effectors.

RNAseq reads from *Foph*-infected cape gooseberry plants were mapped against the *Fol* LS transcriptome and the database of 94 putative effectors described above using CLC Genomics Workbench v7.0 with default parameters, except for the similarity and length fraction, which were decreased to 70% and 80%, respectively, to allow mapping to homologous sequences from the various formae speciales. Homologous transcripts of less than 3 kb in length with at least seven unique *Foph* reads mapped were chosen for further analysis. Their length coverage by *Foph* RNAseq reads was then examined manually to select transcripts with complete or nearly complete mapping coverage for further analysis (Table S2).

PCR screening for the presence of *Fol* effector homologues in *Foph*

In silico identifications of *Fol* effector homologues in *Foph* were verified by PCR amplification from *Foph* genomic DNA using primers SIX1F/SIX1R, SIX7F/SIX7R, SIX10F/SIX10R, SIX_inter1F/SIX_inter1R, SIX_inter2F/SIX_inter2R and Ave1F/Ave1R (Table S2), which were designed to amplify the genomic regions of their counterparts in *Fol*. PCR and sequencing were performed at CORPOICA and the sequences from the PCR products were made available for further analysis.

Bioinformatic analysis

Predicted *Foph* protein sequences were compared with homologues found in other formae speciales using BLASTP searches of the NCBI protein databases and TBLASTN searches of the nucleotide and whole-genome shotgun contig databases. The protein sequences retrieved from these searches were aligned using either the CLUSTALW algorithm in MEGA v.7.0 (Kumar *et al.*, 2016) or MAFFT (https://www.ebi.ac.uk/Tools/msa/mafft/; Katoh *et al.*, 2017). Their phylogeny was analysed using BEAST (Bayesian Evolutionary Analysis Sampling Trees) v1.8.4 (https://beast.community/; Drummond *et al.*, 2012) with default settings, and the resulting phylogenetic trees were visualized using FigTree v1.4.3 (https://tree.bio.ed.ac.uk/software/figtree/) and DensiTree (https://www.cs.auckland.ac.nz/~remco/DensiTree/; Bouckaert and Heled, 2014).

SIX1 sequences were analysed for evidence of diversifying selection using the Fixed Effects Likelihood (FEL) method (Kosakovsky Pond and Frost, 2005, 2005b), the Fast, Unconstrained Bayesian Approximation (FUBAR) method (Murrell *et al.*, 2013) and the Mixed Effects Model of Evolution (MEME) method (Murrell *et al.*, 2012), available at the DataMonkey server (https://www.datamonkey.org/; Kosakovsky Pond and Frost, 2005b).

Generation of *Foph SIX1a* and *SIX1b* vectors for *Fol-*Δ*SIX1* complementation

The coding sequences of *Foph* SIX1a and SIX1b were synthesized and cloned into pUC57 by GenScript (Piscataway, NJ, USA). The open reading frames of SIX1a and SIX1b were amplified from the pUC57 constructs using the primer SIX1-F (for both SIX1a

and SIX1b) and SIX1a-R or SIX1b-R, which introduced *Xba*I sites for binary vector cloning (Table S2). PCR was performed with proof reading Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA) in a reaction volume of 50 μL. The *F. oxysporum* transformation vector pPZP200-pSIX1:GFP (provided by Martijn Rep, University of Amsterdam, Amsterdam, the Netherlands) was used as a backbone to assemble the *SIX1a* and *SIX1b* gene complementation constructs. *Foph* SIX1a and SIX1b were each used to replace the green fluorescent protein (GFP) open reading frame in pPZP200-pSIX1:GFP via *Xba*I cloning to generate the vectors pPZP200-pSIX1:SIX1a and pPZP200-pSIX1:SIX1b (Fig. S10). The correct insertion of each gene in these constructs was confirmed by sequencing.

Fungal strains used for transformation with *ΔSIX1* gene complementation vectors

Fol race 3 isolate #029 (designated *Fol*-WT) and a *ΔSIX1* derivative (designated *Fol-ΔSIX1*) were provided by Martijn Rep (University of Amsterdam, Amsterdam, the Netherlands). *Fol-ΔSIX1* was transformed using *Agrobacterium tumefaciens* strain LBA4404 containing the appropriate binary vector. The protocol for *A. tumefaciens*-mediated transformation was adapted from Mullins and Kang (2001). *Fol-ΔSIX1:SIX1a* and *Fol-ΔSIX1:SIX1b* transformants were selected on Czapek Dox agar supplemented with 75 μg/mL augmentin, 50 μg/mL hygromycin and 100 μg/mL zeocin. Transformants were confirmed by PCR using the primers SIX1p-F/SIX1a.2-R, which amplify a product of 492 bp that corresponds to the *Fol* pSIX1:*Foph SIX1a* junction, and the primers SIX1b-Ter-F/SIX1-Ter-R, which amplify a product of 344 bp that corresponds to the *Foph* SIX1b:*Fol* SIX1 terminator junction (Table S2).

Disease assays

The *Fol-ΔSIX1:SIX1a* and *Fol-ΔSIX1:SIX1b* transformants were tested for pathogenicity, relative to *Fol*-WT and *Fol-ΔSIX1*, on susceptible tomato cultivars M82 and Moneymaker, and on the *Fol* race 3 resistant tomato line IL7-3, which carries the *I-3* resistance gene. Tomato seedlings were inoculated using a modified root-dip method (Mes *et al*., 1999), 11 days after seed sowing. At least 10 plants per line were tested. After removing seedlings from the soil, the roots were washed in water, trimmed and dipped into a spore suspension (5×10^6 conidia/mL) or water (mock inoculation) for 3 min before being replanted. Spores were collected from 5-day-old cultures of *Fol*-WT, *Fol-ΔSIX1* and *Fol-ΔSIX1:SIX1a* or *Fol-ΔSIX1:SIX1b* transformants grown in potato dextrose broth (Difco, Detroit, MI, USA). Inoculated and mock-inoculated plants were kept in a controlled-environment growth room with a 16 h/25 °C day (100 μE/m^2/s) and 8 h/20 °C night cycle for 3 weeks. Wilting symptoms and vascular browning were then recorded and used to calculate disease scores according to the criteria described by Rep *et al.* (2005) and Gonzalez-Cendales *et al.* (2016): 0, healthy plant; 1, slightly swollen or bent hypocotyl; 2, one or two brown vascular bundles in hypocotyl; 3, at least two brown vascular bundles and growth distortion; 4, all vascular bundles were brown and plant either dead or very small and wilted. Differences in the distributions of disease scores between treatments were tested for statistical significance by pairwise two-tailed Mann–Whitney tests (https://vassarstats.net/).

RT-PCR analysis of *fol-ΔSIX1* transformants carrying *Foph SIX1a* or *Foph SIX1b*

Two-week-old susceptible tomato cv. M82 seedlings were inoculated by dipping their roots in a suspension of 5×10^6 conidia/mL of *Fol*-WT, *Fol-ΔSIX1*, *Fol-Δ*SIX1:SIX1a or *Fol-Δ*SIX1:SIX1b, or mock inoculated by dipping in water. Plants were then grown in a controlled-environment growth room with a 25 °C/16 h day (100 μE/m^2/s) and 20 °C/8 h night cycle until collection of samples. Roots of three to four *Fol*-infected or mock-inoculated plants were collected at 3 and 6 dpi, washed with sterile deionized water, pooled in a microcentrifuge tube and frozen in liquid nitrogen ready for RNA extraction.

Frozen root samples were ground in liquid nitrogen and total RNA was extracted using a Plant RNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Total RNA (2 μg) was treated with 2 μL of RQ1 RNase-Free DNase (Promega, Madison, WI, USA) in a reaction volume of 20 μL containing 1 × RQ1 DNAse reaction buffer, followed by an inactivation step at 65 °C for 20 min. Treated RNA (1 μg) was reverse transcribed into cDNA using Superscript III Reverse Transcriptase and an oligo [dT] 12-18 primer (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. PCR (35 cycles) was carried out using MyTaq™ DNA polymerase (BIOLINE, London, UK) in a reaction volume of 15 μL containing 1 μL of cDNA template according to the manufacturer's instructions. Primers RT_SIX1a-F/RT_SIX1a-R for *Fol-Δ*SIX1:SIX1a transformants, and RT_SIX1b-F/RT_SIX1b-R for *FolΔ*SIX1:SIX1b transformants (Table S2) were used to amplify *SIX1a* and *SIX1b* transgenes, respectively. The *F. oxysporum Extracellular Matrix 1* gene (*FEM1*), amplified using the primers RT_FEM1-F/RT_FEM1-R and q_FEM1-F/q_FEM1-R (Table S2), was used as a positive control for fungal gene expression.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Number of *Fusarium oxysporum* f. sp. *physali* (*Foph*) RNA sequencing (RNAseq) reads mapping to *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) effector and FOXM_16306 coding sequences (CDS) and their coverage.

Table S2 Primers used in this research.

Fig. S1 Protein sequence alignments of effectors shared between *Fusarium oxysporum* f. sp. *physali* (*Foph*) and *F. oxysporum* f. sp. *lycopersici* (*Fol*) or *F. oxysporum* f. sp. *medicaginis* (FOXM_16306). Identical residues are indicated by asterisks below the alignment, very conservative substitutions by colons, less conservative substitutions by dots and non-conservative substitutions by blank spaces. *Foph* transcript and protein sequences have been deposited as GenBank accessions MG680406–MG680411.

Fig. S2 Phylogenetic analysis of predicted SIX7, SIX10, SIX12, SIX15, Ave1 and FOXM_16306 protein sequences from *Fusarium oxysporum* f. sp. *physali* (*Foph*) and various other formae speciales of *Fusarium oxysporum*. *Arabidopsis*, *Cercospora*, *Colletotrichum* and *Verticillium* homologues were used as outliers in some of these analyses. Internal node supports are indicated as Bayesian probabilities. Scale bars indicate time in millions of years. *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Foph* sequences are highlighted in green to show their close phylogenetic relationship when both are present.

Fig. S3 Alignment of *Fusarium oxysporum* f. sp. *physali* (*Foph*) and *F. oxysporum* f. sp. *lycopersici* (*Fol*) *SIX7*, *SIX10*, *SIX12* and *SIX15* coding sequences. The *Fol* SIX7, SIX12 and SIX15 sequences used in these alignments are transcript sequences derived from RNA sequencing (RNAseq) data (GenBank accessions MG647014, KU710369 and KY073750, respectively). *Foph* transcript sequences have been deposited as GenBank accessions MG680406–MG680409.

Fig. S4 Alignment of *Fusarium oxysporum* f. sp. *physali* (*Foph*) and *F. oxysporum* f. sp. *lycopersici* (*Fol*) Int-1 and Int-2 nucleotide sequences. *Fol* Int-1 and Int-2 sequences were obtained from the *Fol* 4287 genome sequence available at EnsemblFungi (https://fungi.ensembl.org/index.html) and the National Center for Biotechnology Information (NCBI). Adjacent *SIX7*, *SIX10* and *SIX12* coding sequences have been removed from the *Foph* Int-1 and Int-2 sequences.

Fig. S5 Alignment of *Fusarium oxysporum* f. sp. *physali* (*Foph*) and *F. oxysporum* f. sp. *lycopersici* (*Fol*) *FEM1* nucleotide sequences. The *Foph FEM1* sequence, including adjacent 5′ and 3′ untranslated region (UTR) sequences, was obtained by mapping 76 *Foph* RNA sequencing (RNAseq) reads to the *Fol FEM1* gene sequence (with 100% coverage).

Fig. S6 Identification of two homologues of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) *SIX1* in *F. oxysporum* f. sp. *physali* (*Foph*). (A) RNA sequencing (RNAseq) reads from *Foph*-infected cape gooseberry showing the presence of two *Foph SIX1* sequences mapping to the *Fol SIX1* reference sequence. (B) Sequence chromatogram of the corresponding polymerase chain reaction (PCR) product amplified from *Foph* genomic DNA using the primers SIX1F/SIX1R (Table S2, see Supporting Information),

showing the presence of two *Foph SIX1* sequences. Asterisks indicate the positions of mismatches between *Foph SIX1a* and *SIX1b*.

Fig. S7 Alignment of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) SIX1, *F. oxysporum* f. sp. *physali* (*Foph*) SIX1a and *Foph* SIX1b protein sequences. Identical residues are indicated by asterisks below the alignment, very conservative substitutions by colons, less conservative substitutions by dots and non-conservative substitutions by blank spaces. *Foph SIX1a* and *SIX1b* transcript and protein sequences have been deposited as GenBank accessions MG680404 and MG680405, respectively.

Fig. S8 Alignment of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) SIX1, *F. oxysporum* f. sp. *physali* (*Foph*) SIX1a, *Foph* SIX1b and SIX1 protein sequences from other formae speciales of *F. oxysporum*. The predicted signal peptide sequence is overlined in red and the predicted prodomain sequence defined by a predicted Kex2 cleavage site (highlighted in purple) is overlined in blue. Identical residues are highlighted in black and conservative substitutions are highlighted in grey. Asparagine residues in conserved *N*-glycosylation sites are highlighted in yellow and conserved cysteine residues likely to be involved in disulfide bonding are highlighted in dark blue. A hypervariable region is underlined (dotted line).

Fig. S9 A plot generated using DensiTree of 1001 phylogenetic trees generated using BEAST highlights visually the support for the SIX1 clades shown in Fig. 2.

Fig. S10 Polymerase chain reaction (PCR) screening of *Fol-ΔSIX1* transformants for the presence of the *Fusarium oxysporum* f. sp. *physali* (*Foph*) *SIX1a* or *SIX1b* transgenes. (A, B) Schematic representations of the T-DNAs in the *Foph SIX1a* and *SIX1b* complementation vectors, respectively. Green and red arrows indicate the primer binding sites and amplicon sizes for the *SIX1a* and *SIX1b* transgenes, respectively. (C, D) PCR screening of eight *Fol-ΔSIX1* transformants for the presence of *Foph SIX1a/b* transgenes. Left and right gel images show PCR products (492 bp and 344 bp) of four transformants generated with the *SIX1a* construct and four generated with the *SIX1b* construct, respectively. (C) shows the PCR screening results using crude DNA, whereas (D) shows the PCR screening results using purified DNA from monospore cultures of the transformants tested in (C). PCR product visualization was carried out following electrophoresis in 1.5% agarose gels. Controls included *Fol-ΔSIX1* genomic DNA, pPZP200-pSIX1:SIX1a or pPZP200-pSIX1:SIX1b DNA (pDNA) or no template (H_2O).

Fig. S11 Distributions of disease scores at 21 days post-inoculation (dpi) for individual replicates of pathogenicity tests shown in Figs 3 and 4 for susceptible tomato plants inoculated with *Fol*-WT (WT), *Fol-ΔSIX1* (ΔSIX1), *Fol-ΔSIX1:SIX1a* (SIX1a) or *Fol-ΔSIX1:SIX1b* (SIX1b) transformants. (A) SIX1a transformants 3, 16 or 17 (four replicates with nine or 10 plants per replicate). (B) SIX1a transformants 22, 25 28 or 29 (two replicates with 8–10 plants per replicate). (C) SIX1b transformants 3, 4 or 6 (three replicates with 8–10 plants per replicate.) Data were analysed using pairwise two-tailed Mann–Whitney tests. Treatments with different letters are significantly different at $P = 0.05$.